

Disruption of *YCP4* Enhances Freeze-Thaw Tolerance in *Saccharomyces Cerevisiae*

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Research Article

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Abstract

Objective The aim of this study was to identify genes related to a freeze-thaw tolerance and to elucidate the tolerance mechanism in yeast *Saccharomyces cerevisiae* as an appropriate eukaryote model.

Results In this study, one tolerant strain under exposure to freeze-thaw stress was isolated by screening a transposon-mediated mutant library and the disrupted gene was identified to be *YCP4*. In addition, this phenotype related to freeze-thaw tolerance was confirmed by deletion and overexpressing of this corresponding gene. This mutant strain showed a freeze-thaw tolerance by the reduction in the intracellular level of reactive oxygen species (ROS) and the activation of the *MSN2/4* and *STRE*-mediated genes such as *CTT1* and *HSP12*.

Conclusions Disruption of *YCP4* in *S. cerevisiae* results in increased tolerance to freeze-thaw stress.

Introduction

Freezing and thawing is a type of stress that can cause serious physiological injury to cells, including cell wall and cell membrane damage, DNA and protein degeneration, due to the formation of ice crystals and cell dehydration (Hsu et al. 1979; Mazur 1970). Free radicals produced during the freeze-thaw process are also a major cause of cell damage (Lewis et al. 1997). Although the response of cells to freezing and thawing stress is not known in detail, it is generally accepted that freeze-thaw tolerance is related to factors including growth phase (Werner-Washburne et al. 1993), respiratory metabolism (Lewis et al. 1993), and lipid composition of the membrane (Gélinas et al. 1991). The freeze-thaw stress response is of physiological and industrial importance because it involves other stress response networks, including heat shock and oxidative stress (Cabrera et al. 2020). It has been reported that L-proline protect yeast cells from damage by freezing, or oxidative stress (Takagi et al. 2005). The yeast *Saccharomyces cerevisiae* become more resistant to freeze-thaw stress by adding glycerol (Izawa et al. 2004) and accumulation of trehalose as cryoprotectants (Nakamura et al. 2009), however the mechanisms underlying freeze-thaw tolerance has not been completely understood in the eukaryotes. The aim of this study was to identify genes related to a freeze-thaw tolerance and to elucidate the tolerance mechanism in yeast *S. cerevisiae* as an appropriate eukaryote model (Manning et al. 2002).

Several studies have been reported on freeze-thaw tolerance. Hydrogen peroxide pretreatment could induce freeze-thaw tolerance of yeast cells (Park et al. 1998). Alpha-ketoglutarate enhances freeze-thaw tolerance in *S. cerevisiae* (Bayliak et al. 2018). Freeze-tolerant yeast mutant strains were isolated with freeze-thaw cycles after UV irradiation (Teunissen et al. 2002) and ethyl methanesulfonate (EMS) processing by evolutionary engineering (Cakar et al. 2005). Some yeast strains with freeze-thaw tolerance have been selected from natural sources (Hahn & Kawai, 1990) and constructed by gene manipulations (Shima et al. 1999). In addition, the genes conferring freeze-thaw sensitivity were identified by genome-wide screening of *S. cerevisiae* deletion strains and revealed as genes related to vacuole functions and cell wall biogenesis (Ando et al. 2007). An alternative method is to use disruptive mutants

for direct isolation of strains tolerant to various stress factors (Yazawa et al. 2007; Yoshikawa et al. 2009). I have previously reported the isolation of an organic solvent and heavy metal-tolerant strain of *S. cerevisiae* by screening a transposon-mediated mutant library, respectively (Kim 2016; Kim 2020). A transposon-mediated mutant library has advantages as a means of directly selecting freeze-thaw tolerant strains and construction of mutants harboring up- or down-regulated genes by insertion in the regulatory regions, which is particularly important for lethal genes. In this study, by screening a transposon-mediated disruption mutant library in *S. cerevisiae* L3262, a freeze-thaw resistant strain was obtained and the gene involved was identified.

Materials And Methods

Strains, culture conditions and transposon mutagenesis

The strains and plasmids used in this study are listed in Table 1. *S. cerevisiae* deletion library was provided from Seoul National University (Seoul, South Korea). Yeast strains and *Escherichia coli* DH5 α were cultured according to the previous method (Kim 2016). The mTn3-mutagenized genomic library was digested with *Not*I and transformed into *S. cerevisiae* L3262 by the lithium acetate as previously reported (Livak and Schmittgen 2001).

Freezing and thawing conditions

Yeast cells were cultured on YPD medium at 30°C to exponential phase, were harvested by centrifugation and washed with distilled water. After dilution with 10 mM potassium phosphate buffer (pH 7.0) to an optical density (OD) at 600 nm of 1.0, 0.5 ml were transferred into 1.5 ml eppendorf tubes and frozen at -20°C for 1, 3, and 7 day and then thawed at 0°C for 40 min. Survival was determined by diluting cells into YPD medium at room temperature and plating on YPD plate at 30°C for 2 days. Data was expressed as a percentage of the colony-forming unit (CFU) after freeze-thaw stress treatments compared with no stress conditions.

Isolation of freeze-thaw tolerant mutants and identification of disrupted genes

Yeast transformants were treated under freeze-thaw condition and cultured on YPD plates by spot assay (Kim 2016). Mutant strains that grew faster than wild-type strain were finally selected. All the experiments for selection of freeze-thaw tolerant strains were examined in triplicate. The disruption sites were identified according to the reported method (Livak and Schmittgen 2001). The transposon inserted genes were determined through the *Saccharomyces* Genome Database.

Gene cloning

The open reading frames of identified genes from *S. cerevisiae* L3262 were cloned into pRS316-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for overexpression experiments (Kim 2016). The ORFs with their own promoter and terminator sequences from *S. cerevisiae* L3262 were constructed into pRS316 for complementation experiments (Table 1) (Kim 2016).

Quantitative reverse transcription PCR (qRT-PCR)

All yeast strains were cultivated in YPD medium under freeze-thaw condition. Quantitative reverse transcription PCR (qRT-PCR) was performed using gene specific primers (Table 1) according to the previous method (Kim 2016).

Measurement of intracellular reactive oxygen species (ROS)

The level of intracellular ROS was measured according to the reported method (Madeo et al. 1999) using 2',7'-dichlorodihydrofluorescein diacetate (DCFDA; Molecular probes, Eugene, USA). Exponentially growing yeast cells were treated under freeze-thaw condition. Measurement of intracellular ROS was done as previously described (Kim et al. 2012).

Results And Discussion

Selection of freeze-thaw tolerant mutant strain

Approximately 2000 transposon mediated mutants were tested for freeze-thaw tolerance. One strain that grew faster than wild-type strain was finally selected under freeze-thaw condition. As shown in Fig. 1A, one mutant, designated as TN-F, was more tolerant to freeze-thaw stress than wild type (L3262) and TN-F grew much faster than wild-type strain (Fig. 1B). These results indicate that disrupted gene in TN-F caused tolerance to freeze-thaw stress.

Identification of disrupted gene and contribution to freeze-thaw tolerance

The disrupted gene has been shown in Fig. 2. The insertion site was located in the ORF of *YCP4* in TN-F (Table 2). The effect of disrupting *YCP4* on freeze-thaw tolerance was confirmed using the single-gene deletion library of *S. cerevisiae* BY4741. When the culture of *ΔYCP4* was spot-assayed in a YPD medium under freeze-thaw condition, it showed an improved growth compared to the BY4741 strain (Fig. 3A). *YCP4* is known as one of flavodoxin-like proteins and is predicted to be palmitoylated, a post-translational modification typical of membrane-binding proteins involved in signal transduction (Roth et al. 2006). There have been no reports connecting *YCP4* to a freeze-thaw tolerance in eukaryotic microorganisms. However, it has been reported that the double null mutant of *YCP4* along with *RFS1*, one of flavodoxin-like proteins, had an increased response to oxidative stress (Cardona et al. 2011). In addition, *YCP4* have been studied in regulation of the tumor suppressor PTEN (Kim et al. 2011) and regulated the expression of many genes during the late stages of growth (Cardona et al. 2011), but its exact function has not been revealed yet. In this study, the disruption of *YCP4* clearly showed an improved tolerance to freeze-thaw stress. Therefore, I suggest that *YCP4* may play a role in response and in the regulation of genes related to freeze-thaw stress, although further studies will be required to elucidate the mechanisms related to freeze-thaw tolerance.

Confirmation of tolerance by complementation

Since Mutant strain TN-F acquired a freeze-thaw tolerance by disruption of specific gene, complementation of the relevant gene would create a freeze-thaw sensitive phenotype to the yeast cells. To verify this hypothesis, the respective gene was endogenously expressed by cloning the amplified DNA fragments estimated to include ORF, their own promoter, and their own terminator (ORF \pm 700 bp) into pRS316 and transformed into TN-F. The reference strain was obtained by transforming pRS316 containing nothing. The tolerance of the complemented strain was significantly reduced compared with that of the reference strain (Fig. 1B). In addition, the ORF of *YCP4* was amplified by PCR, constructed into pRS316-GAPDH for an overexpression, and transformed into L3262. The overexpressed yeast strain (L3262/*YCP4*) was more sensitive to freeze-thaw stress (Fig. 1C). In addition, the gene dosage of *YCP4* was examined by qRT-PCR. The expression patterns of *YCP4* are higher by the control of the GAPDH than by that of their own promoter (Fig. 4). Therefore, these results suggest that the sensitive phenotype to freeze-thaw stress may be determined by the expression level of the *YCP4* gene.

Intracellular ROS at freeze-thaw stress

Several studies have been reported that oxidative stress is a major cause of yeast cell damage, which is responsible for accumulation of ROS during freeze-thaw stress (Nakagawa et al. 2013; Park et al. 1998). Therefore, I investigated whether TN-F could reduce the intracellular level of ROS that accumulated upon exposure to freeze-thaw stress. Compared to the control strain (L3262), the level of ROS signal was approximately 55% lower in the TN-F mutant under freeze-thaw stress condition (Fig. 5), indicating that the reduction in ROS was associated with the freeze-thaw tolerant phenotype. Next, it has been reported that the overexpression of the transcriptional activator, *MSN2* conferred the tolerance to freeze-thaw stress (Sasano et al. 2012). In contrast, the disruption of the *MSN2* gene caused sensitivity to freeze-thaw stress in a laboratory yeast strain (Izawa et al. 2007). In addition, it has been known that the stress-response transcription factors, including the *MSN2/4* bind to stress response elements (STRE) within the promoters of stress-mediated genes such as *CTT1*, *HSP12* under various stress (Causton et al. 2001; Martínez-Pastor et al. 1996). Thus, I compared the transcript levels of *MSN2*, *MSN4*, *CTT1*, and *HSP12* between the L3262 and TN-F strains. As shown in Fig. 6, the mRNA levels of *MSN2*, *MSN4*, *CTT1*, and *HSP12* in TN-F increased compared with those in the control strain (L3262) under freeze-thaw stress. Without any freeze-thaw stress, the mRNA patterns of corresponding genes in all strains were not changed. In *S. cerevisiae*, double deletion mutant $\Delta msn2 \Delta msn4$ has hypersensitive phenotype to carbon source starvation, heat shock, osmotic and oxidative stresses (Estruch and Carlson 1993). In addition, it has been reported that the expression of *CTT1* gene was increased in double deletion mutant $\Delta ycp4 \Delta rfs1$, referred to as oxidative stress tolerance (Cardona et al. 2011). In this study, the disruption of *YCP4* increased freeze-thaw tolerance with the activation of *MSN2/4* and STRE-mediated genes such as *CTT1* and *HSP12*. Therefore, these results suggest that the disruption of *YCP4* may contribute to a freeze-thaw tolerance through ROS scavenging by the expression of the *MSN2/4* and STRE-mediated genes such as *CTT1* and *HSP12*. Further studies on an *YCP4*-mediated metabolic regulation are required to elucidate the mechanisms of freeze-thaw tolerance. In conclusion, the characteristics of *YCP* gene will contribute to the application of freeze-thaw processes

including frozen dough baking and cryopreservation and to provide clues on freeze-thaw tolerance in higher eukaryotic organisms.

Declarations

Acknowledgements

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Tables

Due to technical limitations, tables are only available as a download in the Supplemental Files section.

Figures

Fig. 1

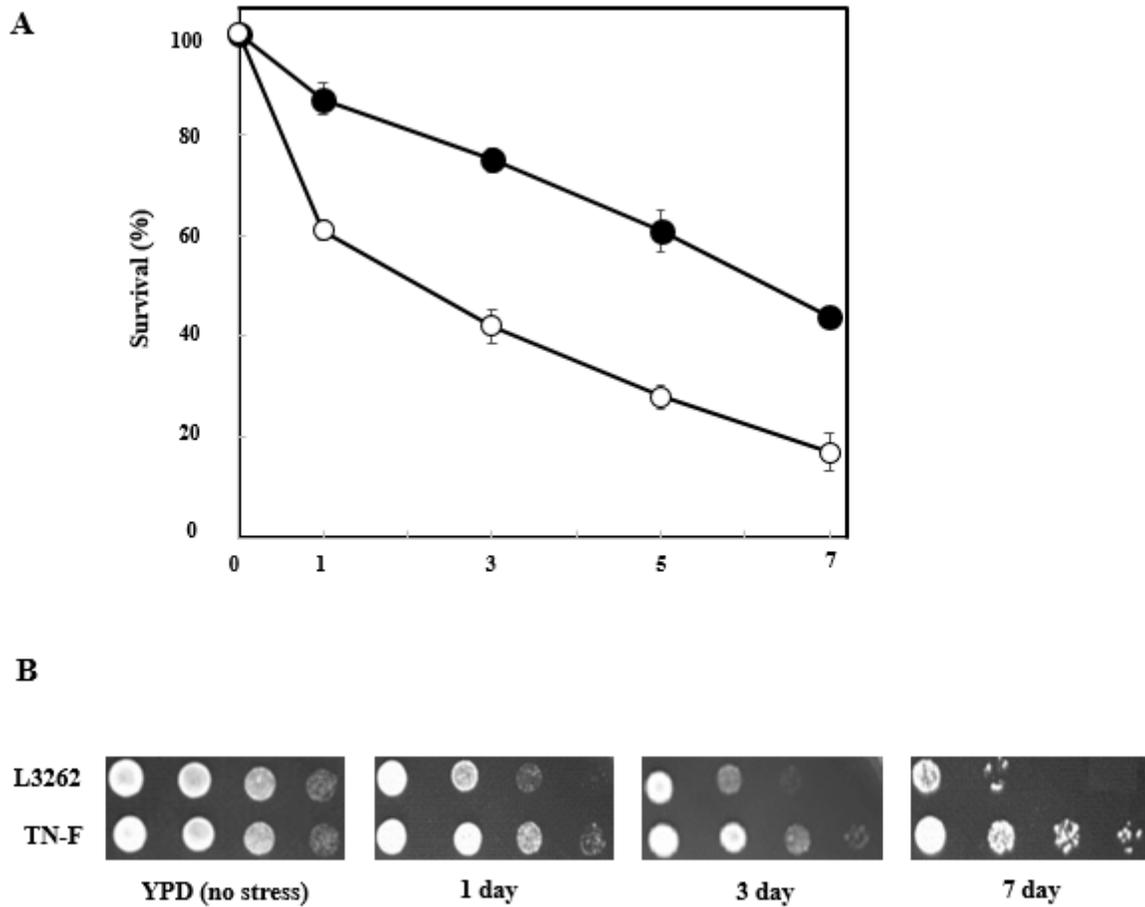


Figure 1

Identification of freeze-thaw tolerance strain. A Viabilities were determined with L3262 (open circle) and TN-F (closed circle). Survival percent was expressed relative to the initial viability. B Tenfold serial dilutions of L3262 and TN-F were spotted on YPD plates under freeze-thaw stress conditions (Materials and Methods)

Fig. 2

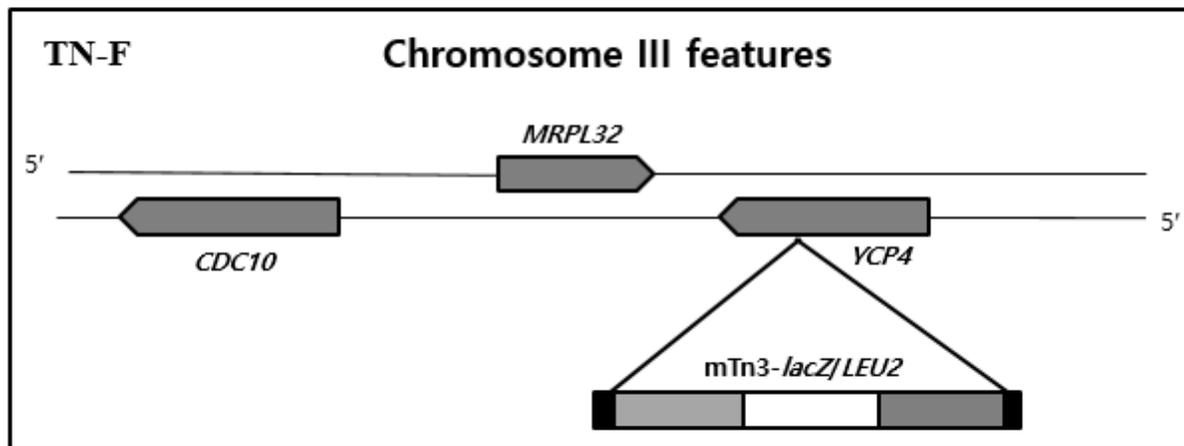


Figure 2

Determination of transposon insertion site. Transposon insertion site was determined by sequencing of recovered transposons from freeze-thaw tolerant strain. Surrounding genetic loci are shown

Fig. 3

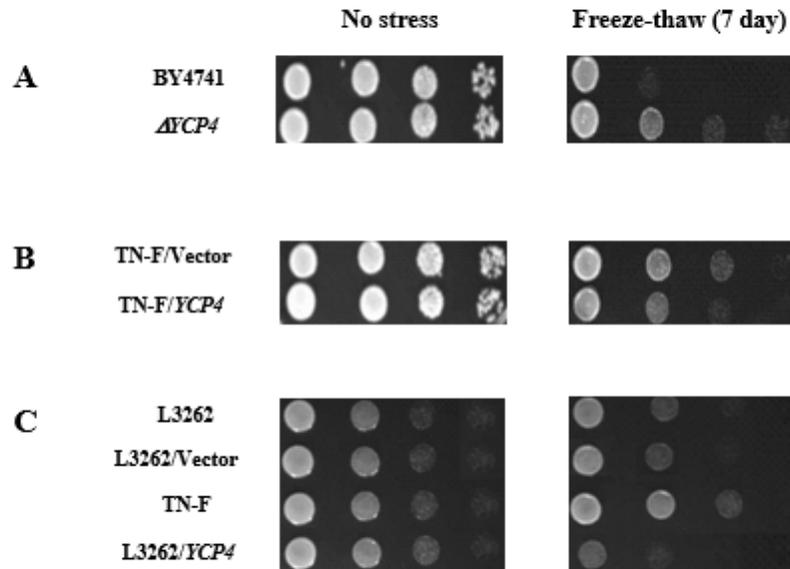


Figure 3

A Freeze-thaw tolerance of single gene knockout mutants. BY4741 were used as control. B Restoration of stress sensitivity by complementation. Control strains were constructed by transforming pRS316 into TN-F. C Effect of overexpression on stress sensitivity. Control strains were constructed by transforming pRS316-GAPDH into L3262. Tenfold serial dilutions were spotted on YPD plates under freeze-thaw stress conditions (Materials and Methods)

Fig. 4

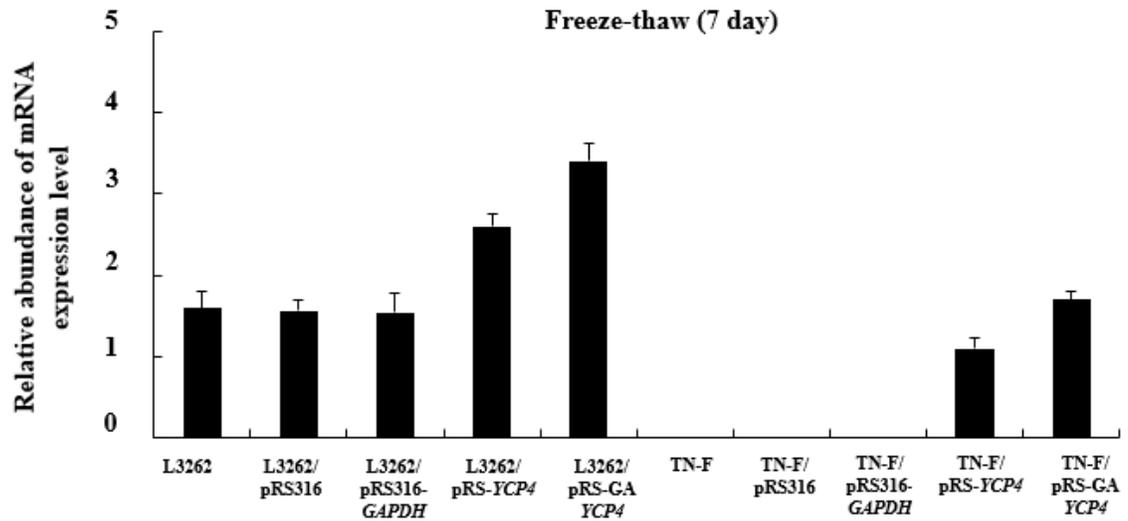


Figure 4

Dosage effect of YCP4. The effect was determined by quantitative reverse transcription PCR. Values were normalized to glyceraldehyde 3-phosphate dehydrogenase before calculating changes. Control strain was TN-F

Fig. 5

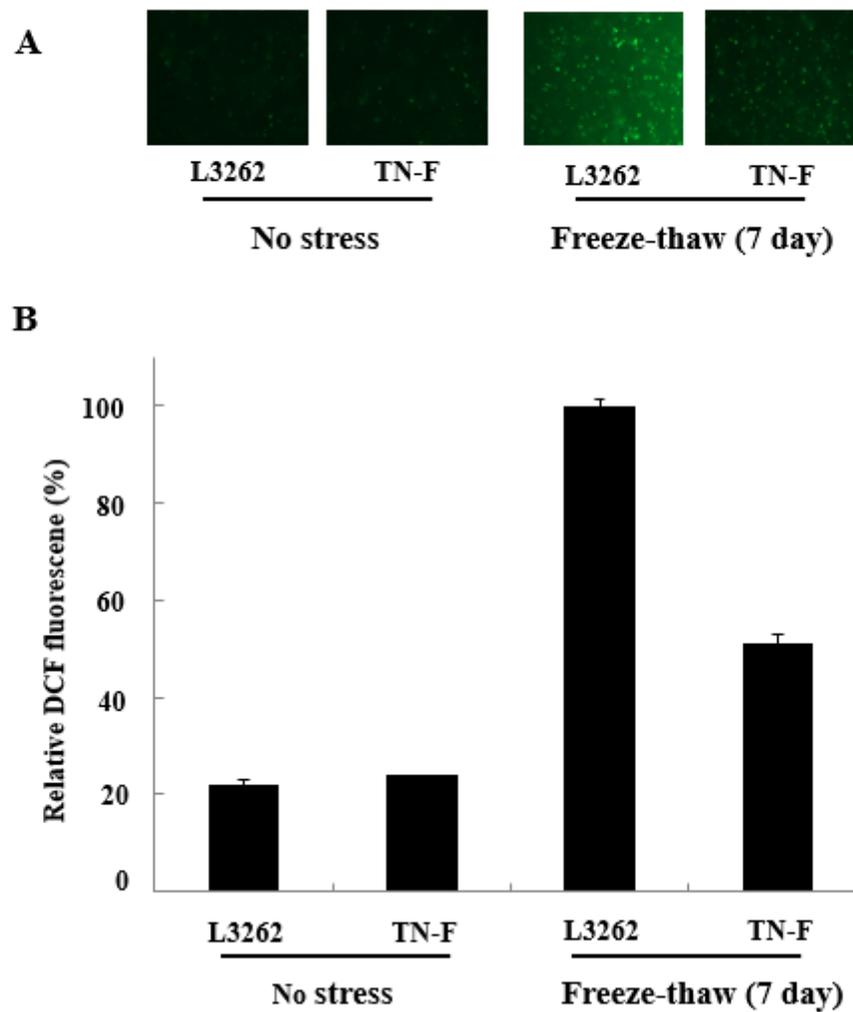


Figure 5

ROS accumulation in TN-F. A Representative images of L3262 and TN-F were taken at 8 h after culturing under freeze-thaw stress conditions. B Relative DCF fluorescence intensity was measured from the images of A by using NIH ImageJ, version 1.61. Averages of three independent values were represented relative to the value of L3262 treated under freeze-thaw stress conditions (Materials and Methods)

Fig. 6

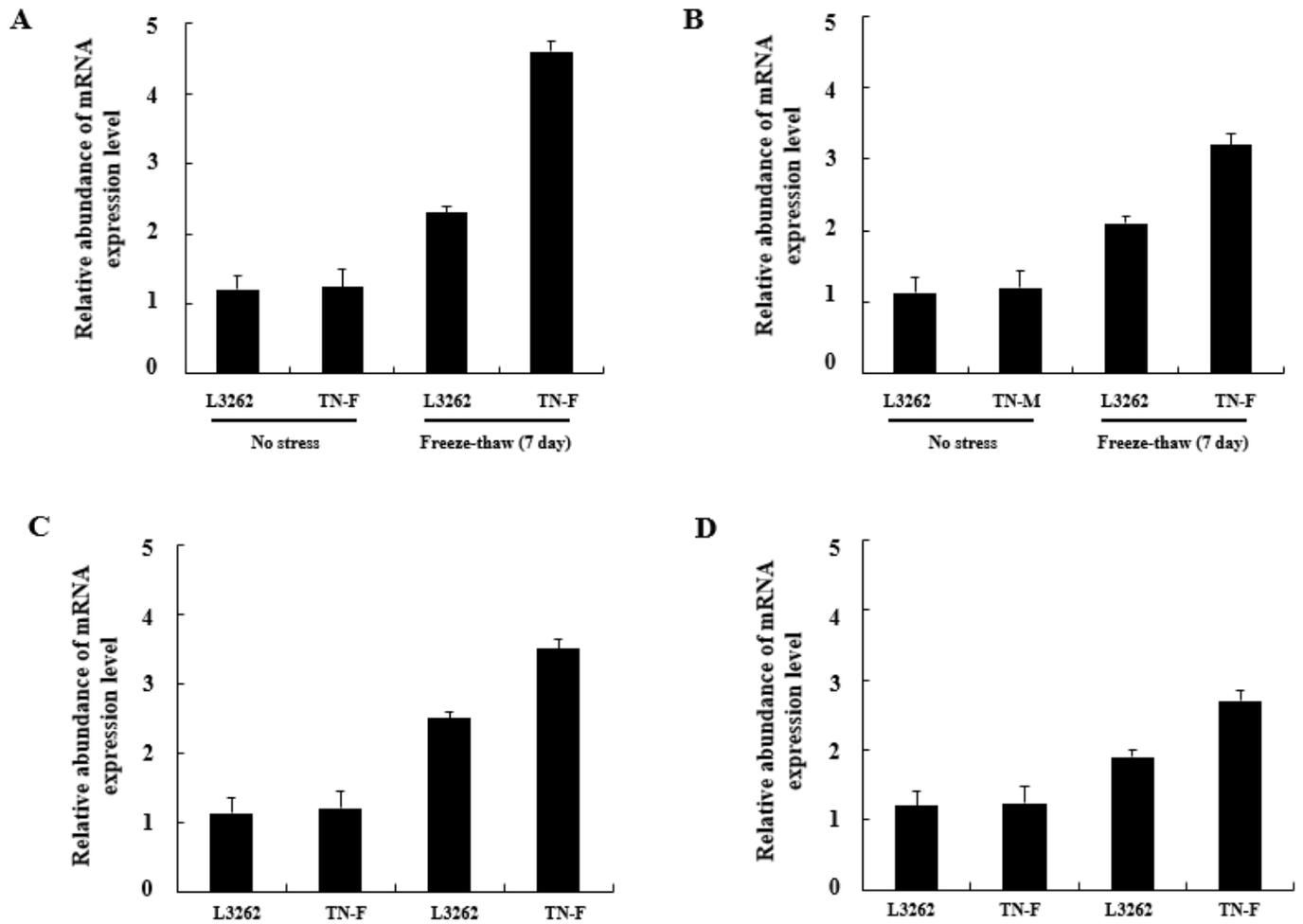


Figure 6

Dosage effect of A MSN2. B MSN4. C CTT1. D HSP12. The effect was determined by quantitative reverse transcription PCR. Values were normalized to glyceraldehyde 3-phosphate dehydrogenase before calculating changes and represented relative to the value of L3262 or TN-F untreated with freeze-thaw (no stress) respectively

Supplementary Files

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